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A comparative study of the pulmonary effects of NO_2 in the rat and hamster

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Summary. A study of the response of rat and hamster to nitrogen dioxide (NO_2) under identical conditions has been undertaken. Exposure to 20 parts/10^6 NO_2 for 24 h produced a mild cytotoxic effect on the terminal bronchiole and proximal alveoli in the rat, whereas the hamster developed a moderate to severe bronchiolitis and alveolitis. Electron microscopic examination of tissue sections showed accumulation of surfactant in lamellar bodies of the alveolar type II cell in the rat but not in the hamster, whereas in the hamster Clara cells were observed in mitosis. Increased levels of surfactant isolated from whole lung homogenates by sucrose gradient centrifugation were found in both rat and hamster. In contrast surfactant isolated from bronchiolo-alveolar lavage was increased only in the hamster. The results suggest that caution must be exercised in the choice of animal model in investigations aimed at the understanding of the toxicological effects of nitrogen dioxide in man.

Keywords: nitrogen dioxide, cytotoxicity, bronchiolitis, alveolitis

The toxic effects of nitrogen dioxide (NO_2) on the lung have been studied extensively over the past 20 years (Bils & Christie 1980), but results have demonstrated a considerable degree of variability which is heavily influenced by the species studied, the magnitude of the dose and the duration of administration. For example, 52 parts/10^6 NO_2 was found to have very little effect on beagle dogs (Carson *et al.* 1962), while a dose as small as 4 parts/10^6 was found to induce hyperplasia in the epithelium of the terminal bronchiole of the rat (Freeman *et al.* 1969).

Of all the species studied, the rat has been used most commonly and much of the data pertaining both to the mode of action of NO_2 on the lung and the responses of lung cells after toxic insult (Evans *et al.* 1978; De Nicola *et al.* 1981; Akino & Ohno 1981) have been derived from this species. The object of the present study, part of a larger investigation into the effects of air pollutants on lung morphology and function, has been to compare the response of the rat with that of a second species, the hamster, to NO_2 with a view to defining more precisely the extent and cause of any species variation in response.

The effects of acute exposure to NO_2 on lung surfactant levels was examined together with an assessment of the changes in terminal bronchioles and alveoli and the free-lying pulmonary cell population.

Materials and methods

Animals. Female Sprague-Dawley rats (100–120 g, Olac (1976) Ltd, Bicester, Oxon,

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UK) or female DSN hamsters (80–100 g, Intersimian, Abingdon, Oxon, UK) were randomly assigned to their holding cages on receipt. Exposure to NO_2 in air at 20 parts/10^6 for 24 h was carried out in glass chambers with an internal capacity of 140 litres. Control animals were exposed in separate chambers for an equivalent period of time to air given at a similar gas flow rate (0.5 l/min per animal). Nitrogen dioxide concentration was continuously monitored using an Ecoylser 3000 electrochemical analyser (Analysis Automation Ltd, Oxford, UK). Throughout the period of exposure the concentration of NO_2 administered was 19.88 ± 0.49 parts/10^6. Separate groups of animals were used for determination of pulmonary surfactant levels, examination of lung tissue and characterization of the bronchiolo-alveolar cell population. All animals were killed within 1 h after exposure by an intraperitoneal injection of Sagatal (0.6 ml/100 g bodyweight).

Surfactant preparation and analysis. Pulmonary surfactant was isolated from whole lung (total surfactant) or from lavage fluid (extracellular surfactant). For whole lung preparations lungs were perfused prior to homogenization to remove blood with 15 ml physiological isotonic saline at 37°C over 1 min, using a constant velocity mechanically driven syringe. Extracellular surfactant levels were prepared from samples obtained after bronchiolo-alveolar lavage with 8×2 ml physiological isotonic saline at 37°C. Surfactant was isolated from either homogenates or lavage fluid by sucrose gradient centrifugation as described by Gratwohl *et al.* (1979). Phospholipid phosphorus was determined by the method of Allen (1940) and phospholipid class composition of extracted phospholipids as described by Cottrell *et al.* (1983) using ^{31}P NMR. Protein was assayed by the method of Lowry *et al.* (1951) in the presence of 0.05% sodium dodecyl sulphate using bovine serum albumin as standard.

Ultrastructural examination. The lungs were fixed by intratracheal instillation of 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) at a pressure of 30 cm of water. After full inflation the trachea was firmly tied and the lungs were left to fix *in situ* for 1 h before removal. After removal the lungs were immersed in excess fixative and allowed to harden fully for a further 3 days. Mid-coronal slices were taken from the left and right lobes and post-fixed in 1% osmium tetroxide in 0.1 M phosphate buffer, dehydrated through graded concentrations of ethanol and embedded in a 1:1 mixture of Agar 100:araldite epoxy resin. Sections were cut at 2–3 μm on a Reichart–Jung 'Autocut', microtome, stained with 1% toluidine blue and examined by light microscopy. Selected regions were cut at a thickness of 900–1100 nm on a LKB Ultratome III ultramicrotome, stained with uranyl acetate and lead citrate and examined in a JOEL 100 CX transmission electron microscope.

Cell collection and culture. Bronchiolo-alveolar cells were recovered from the lungs by repeated lavage with Joklik (Gibco, UK) supplemented with 12 mM lignocaine (Sigma Ltd, UK) and 10% fetal calf serum (FCS, Gibco) according to the method of Miller & Foster (1981). Samples were taken for determination of cell yield and population characteristics of the lavaged population. Viability was determined by trypan blue dye exclusion. Macrophage cell concentration was adjusted to 1×10^6/ml and 1-ml aliquots were applied to sterile glass coverslips (12 mm diameter) in disposable Linbro trays, (Flow Labs, UK). The cells were allowed to adhere to the glass substrates for up to 45 min at 37°C in a standard CO_2 tissue culture incubator. They were then washed vigorously and incubated for a further 2 h in RPMI-1640 containing 10% FCS.

EA cells. Sheep red blood cells (SRBC) were sensitized by incubating equal volumes of a 5% suspension of SRBC in phosphate-buffered saline (PBS) and either a 1:600 dilution of rabbit anti-sheep haemolytic serum (Well-

come Research Labs, Beckenham, UK) or 1:100 rat anti-sheep serum for 30 min at 37°C. For rosette determination a 1% suspension of EA preparation was incubated with washed macrophage cultures for 30 min at 37°C, thoroughly rinsed and fixed in Bouins' fixative and stained with haematoxylin and eosin for light microscopy. The adherence of three or more SRBC to a macrophage constituted a rosette and a total of 200 macrophages were counted per cover slip. Each determination was performed in duplicate.

Results

Increased levels of pulmonary surfactant were observed in both rat and hamster whole lung homogenates following NO_2 exposure (Fig. 1). However, the quantity of surfactant material (which contains both phospholipid and protein components) recoverable by a limited lavage from treated animals of the two species differed considerably (Fig. 2). The extracellular surfactant levels obtained from the exposed hamsters were appreciably greater than those obtained from control

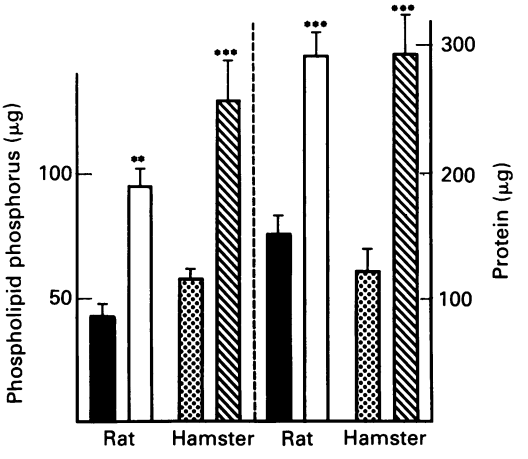

Fig. 1. Effect of 24-h exposure to 20 parts/10^6 NO_2 on rat and hamster whole lung surfactant phospholipid phosphorus and protein. ■, ⊠, Control; □, ▨, treated. Results are expressed as mean ± SE for eight animals as μg per animal. Significance assessed by Student's *t*-test comparing with appropriate control: **P < 0.01; ***P < 0.001.

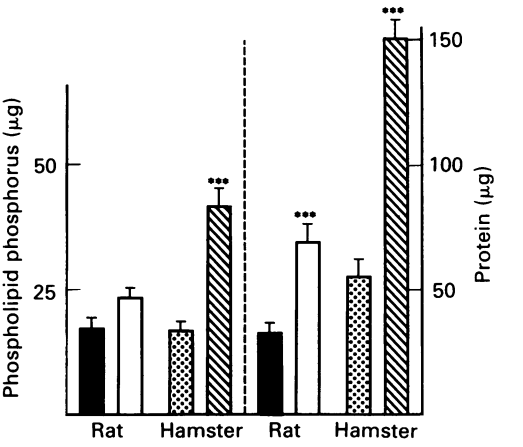

Fig. 2. Effect of 24-h exposure to 20 parts/10^6 NO_2 on rat and hamster lavagable surfactant phospholipid phosphorus and protein. ■, ⊠, Control; □, ▨, treated. Results are expressed as mean ± SE for eight animals. ***P < 0.001.

animals, while only a small difference was found in extracellular surfactant levels obtained from exposed rats compared to untreated controls. ^{31}P NMR analysis of pooled surfactant samples from these experiments did not suggest any significant changes in the phospholipid composition of surfactant isolated from control or exposed animals of either species (data not shown).

Differences in response to NO_2 exposure by rat and hamster were further demonstrated when lungs were examined by light and electron microscopy. Examination by light microscopy of rat lungs showed a smoothing of the normally irregular profile of the terminal bronchiole, the irregularity in control rats being due to the domed cytoplasm of the Clara cells.

In ultrastructural sections cellular changes were clearly seen in the bronchiolar epithelium where the Clara cells showed a considerable reduction in secretory granules and the ciliated cells exhibited a marked loss of cilia (Fig. 3) and increased numbers of autophagic vacuoles. This was in marked contrast to that in the control rat (Fig. 4). In the alveoli, the type I cells showed cytoplasmic thickening and swelling of organelles

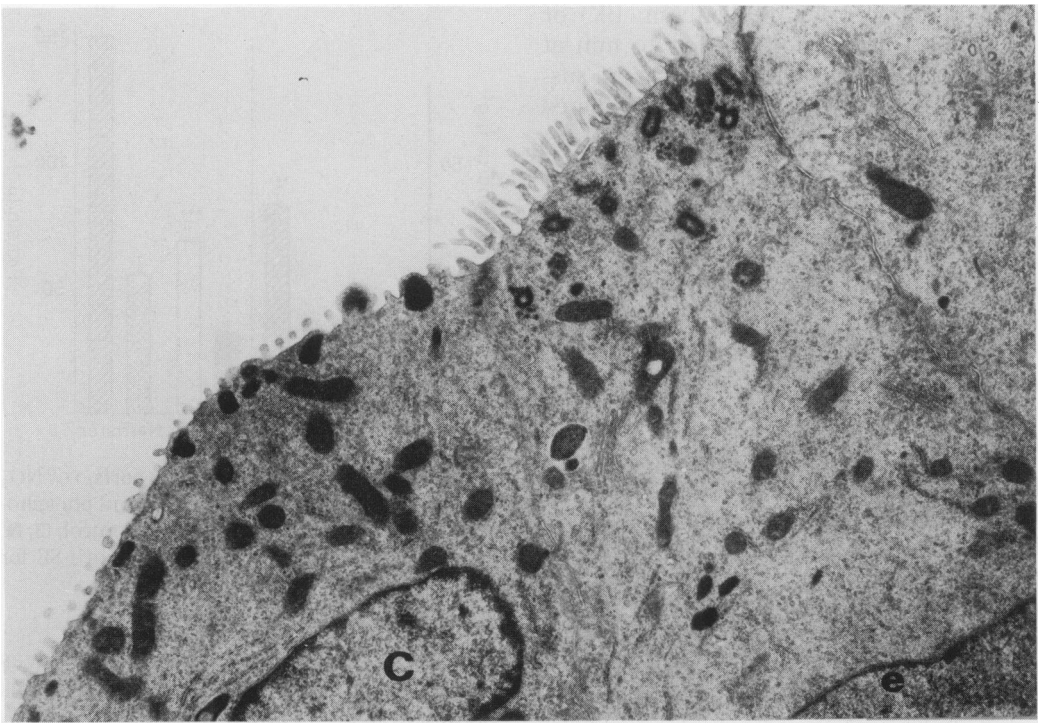

Fig. 3. Electron micrograph of respiratory bronchiole area from a rat treated for 24 h with NO_2, showing flattening of Clara cell (c) and deciliation of ciliated epithelial (e) cell. × 8700.

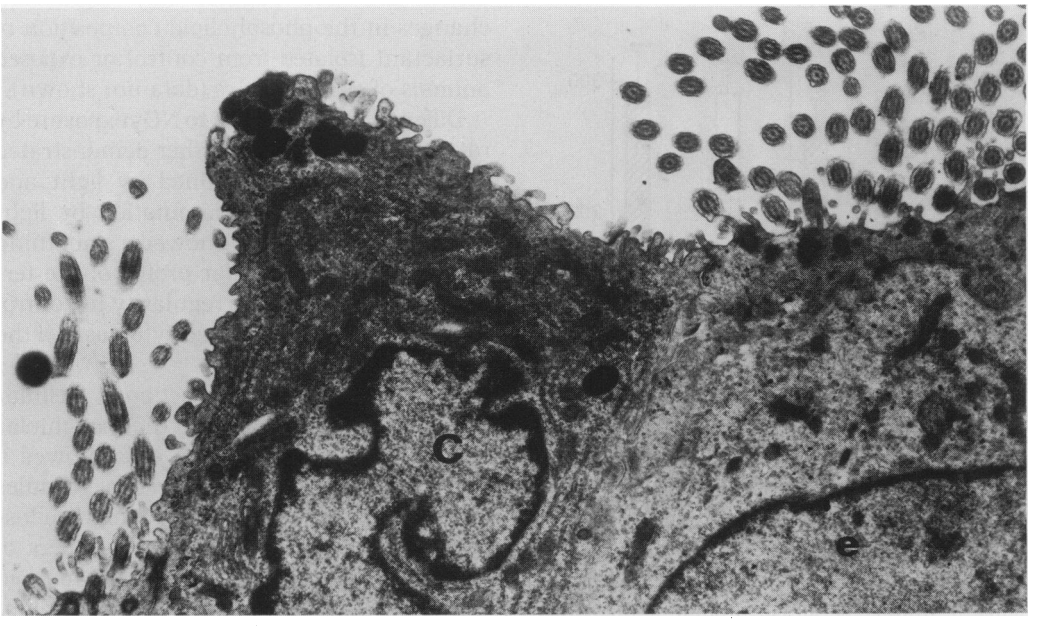

Fig. 4. Electron micrograph of the respiratory bronchiole showing Clara cell (C) and ciliated cell. Untreated rat lung. e, Epithelial cell. × 9000.

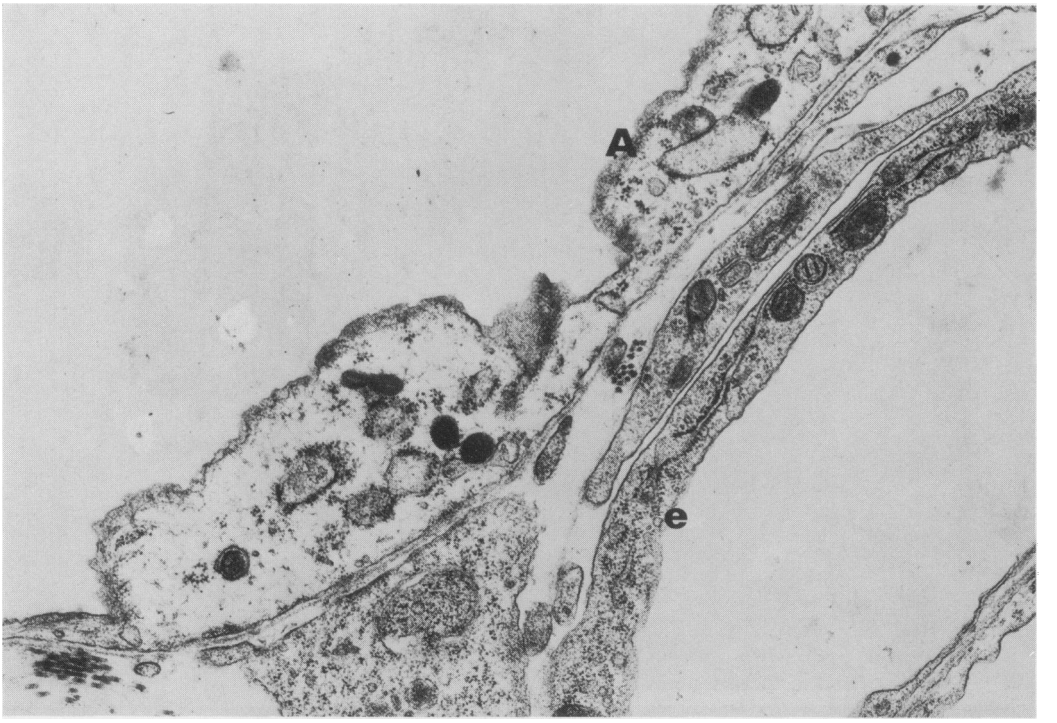

Fig. 5. Electron micrograph of type I alveolar cell (A) from a rat treated for 24 h with NO_2, showing cytoplasmic swelling. e, Epithelial cell. × 17 500.

although the alveolar endothelial cells were normal (Fig. 5). In contrast many of the type II cells were observed containing very large secretory granules in their cytoplasm (Fig. 6) and an increased number of cells were observed in mitosis.

Examination of hamster lungs showed far more severe changes including desquamation of the epithelial lining of the terminal regions of the bronchioles, the lumen of which contained a cellular exudate consisting of necrotic epithelial cells, a mononuclear and neutrophil infiltrate and free red blood cells (Fig. 7). Regions less severely affected showed a smoothing of the normally irregular profile of the terminal bronchiole, which in control animals was very similar to that seen in control rats. The alveoli proximal and adjacent to the bronchioles also showed areas of cell exudate containing red blood cells, neutrophils and mononuclear cells.

In ultrastructural studies the majority of ciliated cells were deciliated and the normally domed cytoplasm of the Clara cell (Fig. 8) was also lost so that the bronchiolar epithelium was uniformly flattened. A good many Clara cells were however observed in mitosis and a few remaining domed cells showed an increase in the number of cytoplasmic granules (Fig. 9). In the bronchiolar lumen large deposits of fibrin were also frequently observed. In the alveoli, type I cells showed similar changes to those seen in the rat, with a swelling of their cytoplasm and vesiculation of the cytoplasmic organelles. The type II alveolar cells and the alveolar endothelial cells appeared normal in spite of the large numbers of free red blood cells and fibrin frequently seen in the air spaces and lumina of the bronchioles.

Differing effects of NO_2 exposure on the bronchiolo-alveolar cell population obtained from rat and hamster were also evident. In

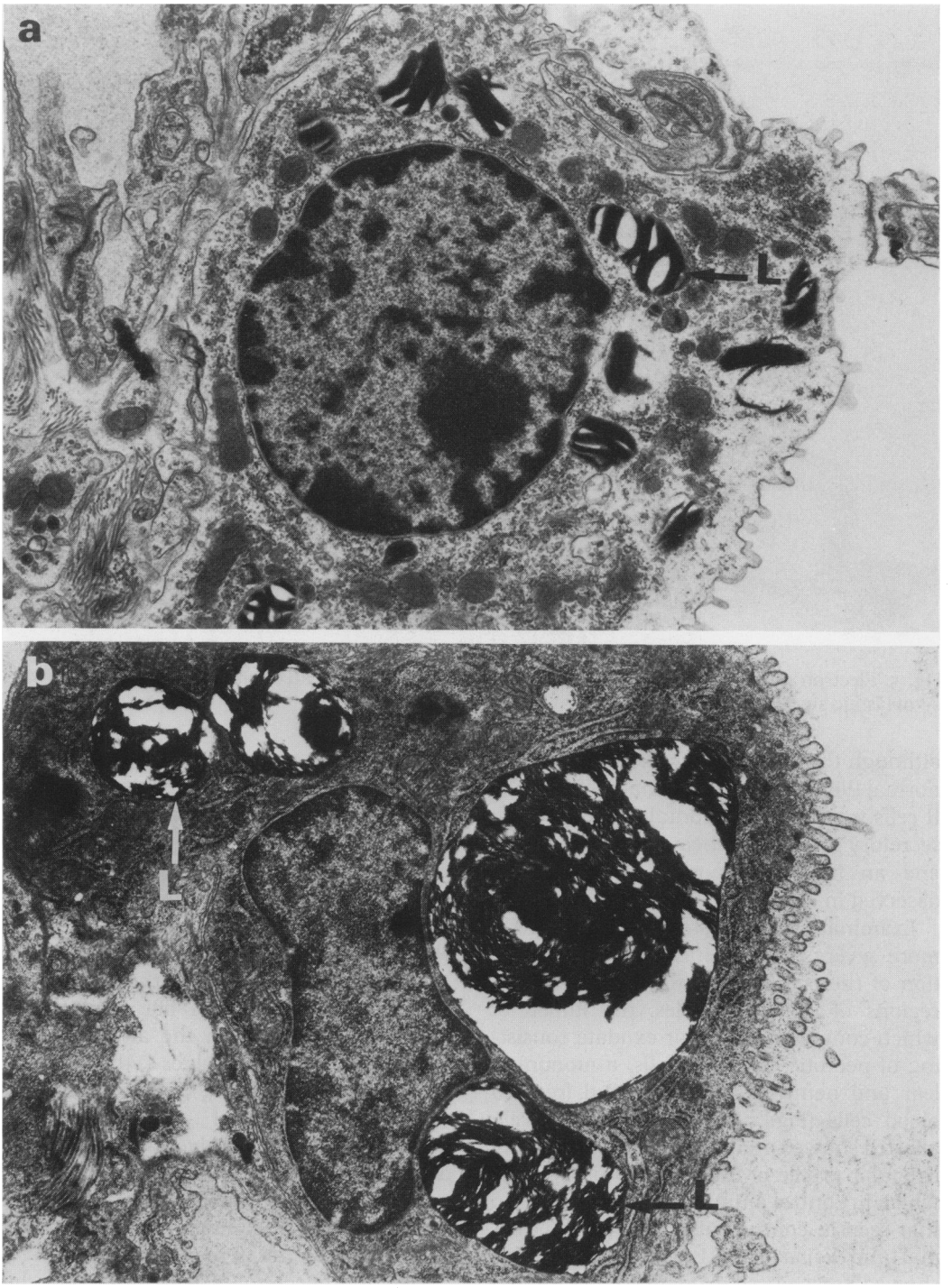

Fig. 6. (*a*) Electron micrograph of type II alveolar cell showing cytoplasmic lamellar bodies (L). Untreated rat lung. × 11 500. (*b*) Electron micrograph of type II alveolar cell from a rat treated for 24 h with NO_2 showing an accumulation of grossly enlarged lamellar bodies (L). × 11 000.

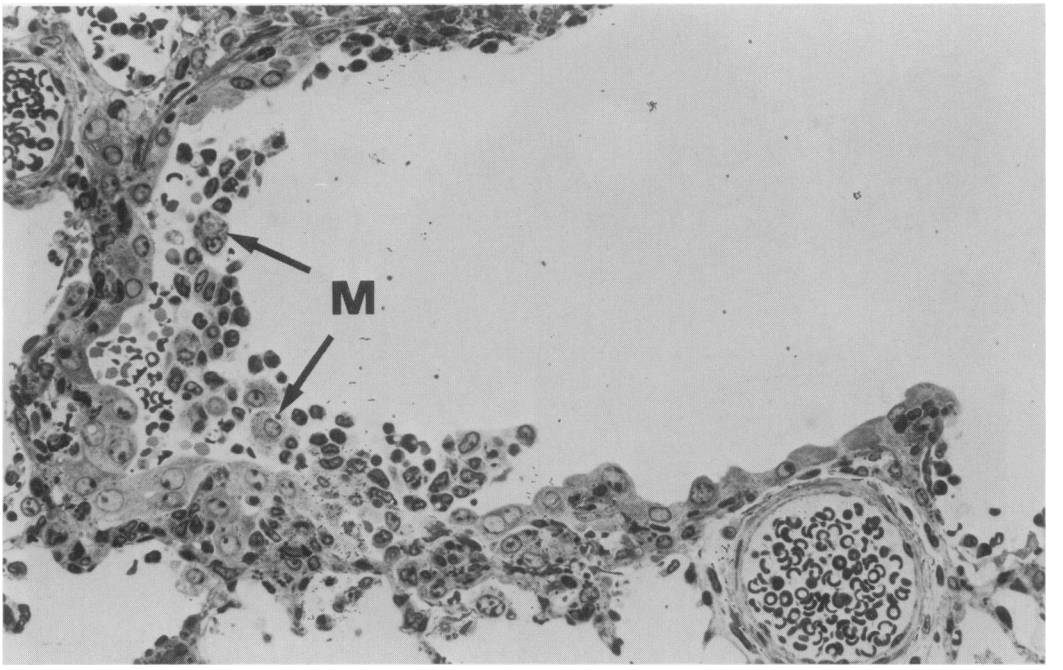

Fig. 7. Light micrograph of respiratory bronchiole region of a hamster treated for 24 h with NO_2, showing cellular exudate in the bronchiolar lumen consisting of macrophages (M), red blood cells and necrotic epithelial cells. ×4000.

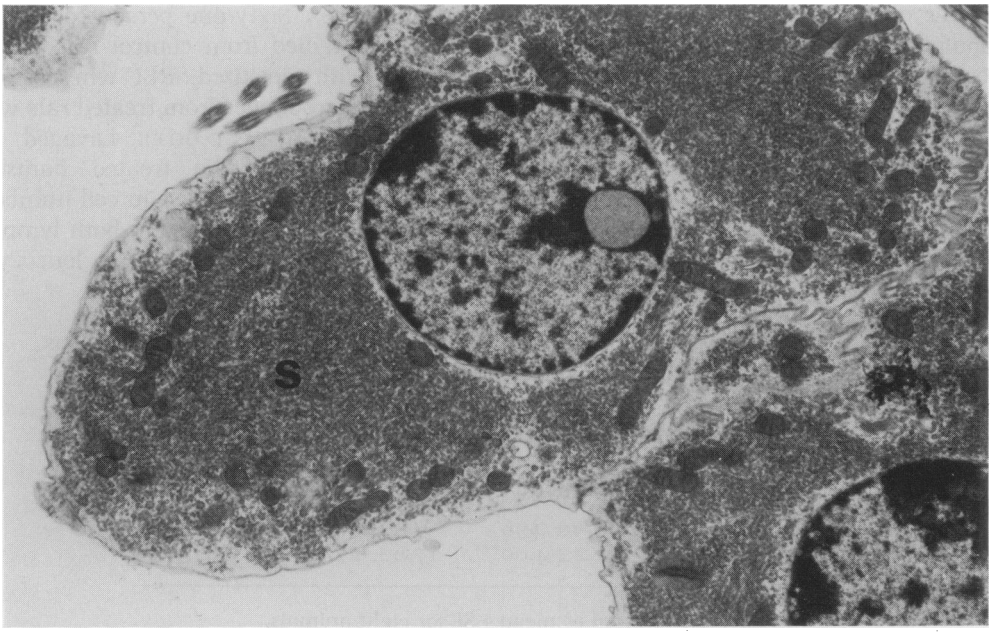

Fig. 8. Electron micrograph of Clara cell from an untreated hamster showing smooth endoplasmic reticulum (S) with few secretory granules. ×6100.

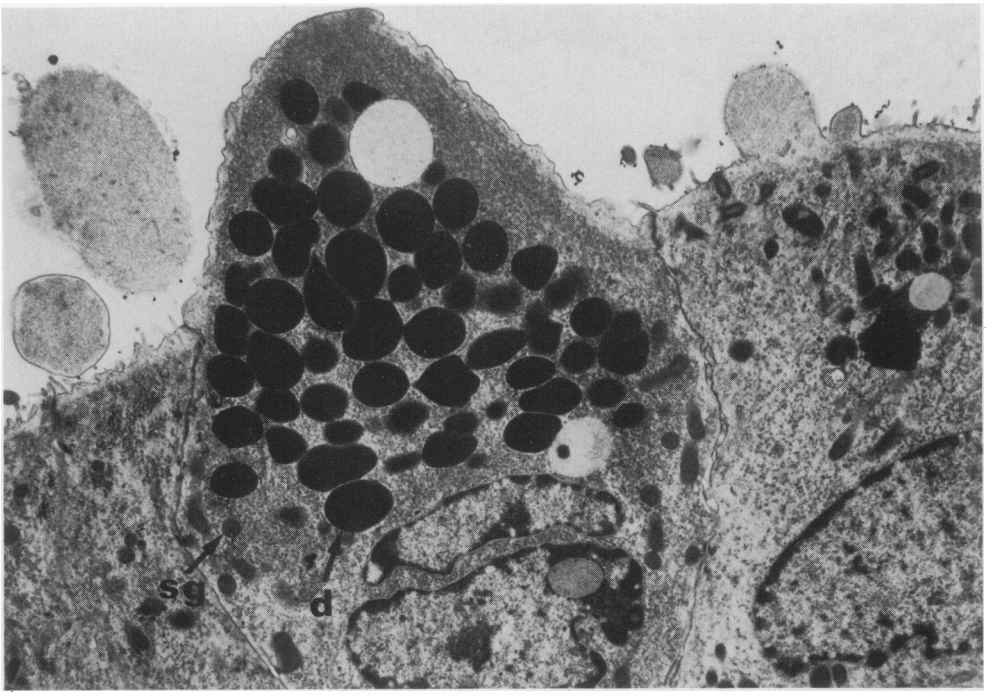

Fig. 9. Electron micrograph of Clara cell from hamster treated for 24 h with NO_2, showing electron-dense droplets (d) in the cytoplasm. sg, Secretory granule. × 5600.

the rat no alteration in the lavaged cells' population was found between control and treated animals. In both groups of animals the cells consisted of 96–98% macrophages (Table 1). Vacuolation of the cytoplasm was observed in some macrophages from treated rats, however, and significant differences were found in the ability of the cells to form EA rosettes compared to control (untreated) macrophages. Sixty-one percent of macrophages obtained from control rats formed rosettes with sensitized SRBC whereas only 31% of macrophages from treated rats were rosetting cells ($P<0.001$). Lavaged cell populations from the treated hamsters showed a marked increase in cell numbers, due mainly to an increase in both lymphocytes and polymorphonuclear leucocytes

Table 1. Effect of 24-h NO_2 exposure on composition of rat or hamster lavage cell populations

	% macrophage	% lymphocyte	% neutrophil
Untreated rat	98.6±0.21	1.0±0.20	0.2
Treated rat	95.9±1.28	1.4±0.38	1.7±0.89
Untreated hamster	86.9±0.96	9.9±0.66	2.3±0.69
Treated hamster	41.2±4.00***	17.6±6.7*	36.8±6.08***

Results are expressed as mean±SE for eight animals.

Significance assessed by Student's *t*-test comparing with appropriate control: *$P<0.05$; ***$P<0.001$.

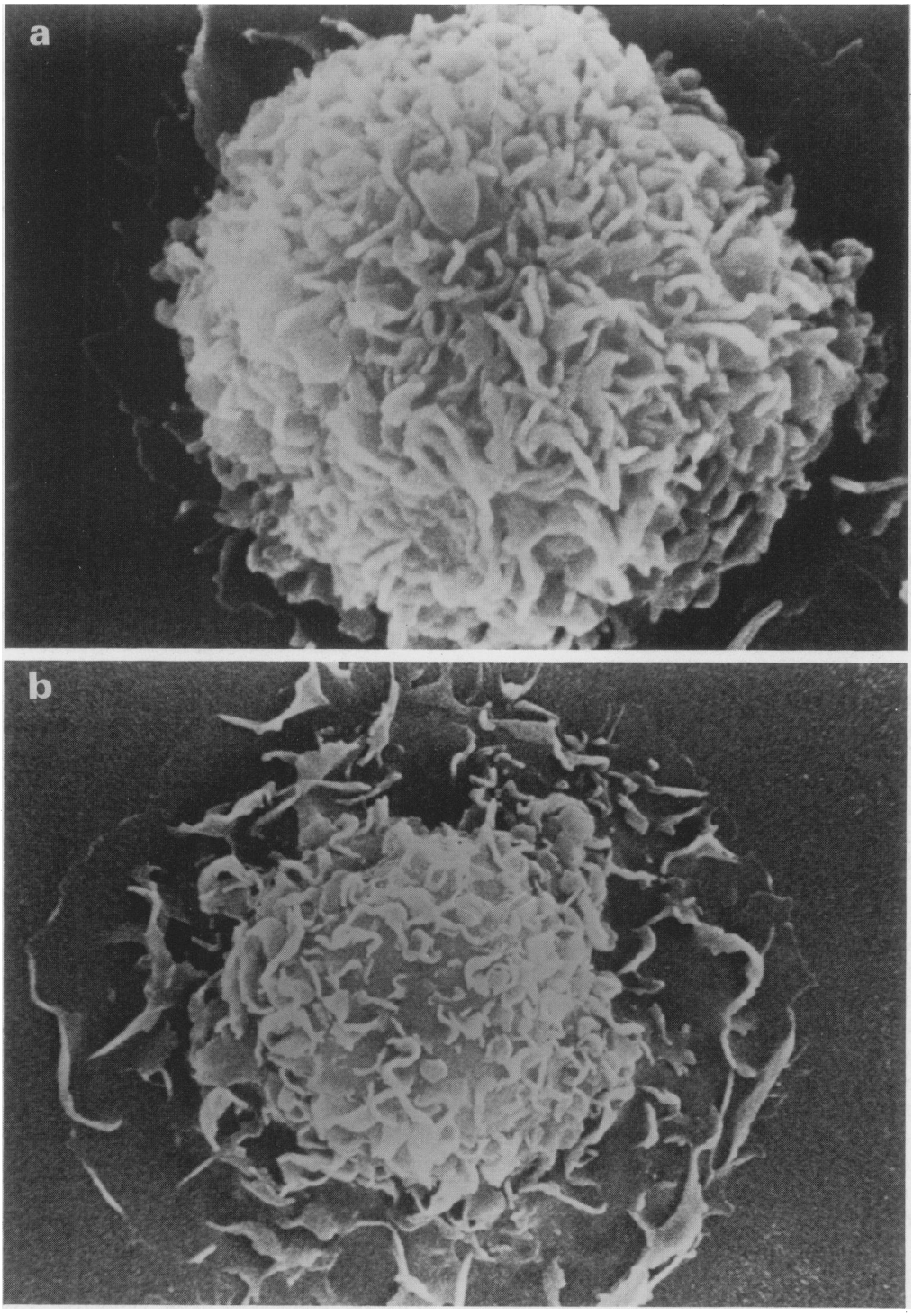

Fig. 10. (*a*) Scanning electron micrograph of alveolar macrophage from untreated hamster. (*b*) Scanning electron micrograph of alveolar macrophage from hamster treated for 24 h with NO_2.

present in the lavage fluid (Table 1). These macrophages were able to spread more extensively on glass substrates (Fig. 10) and exhibited an increased ability to bind sensitized SRBC compared to control macrophages with the number of rosetted cells increased from 55 to 68%. Any toxic effect of NO_2 on hamster alveolar macrophages was thus masked by the inflammatory reaction occurring as a consequence of alveolar capillary damage.

Discussion

Several recent publications have described in detail the morphological and biochemical changes which occur in the lung following acute exposure to NO_2 (Yuen & Sherwin 1971; Guidotti 1980; De Nicola *et al.* 1981; Brown *et al.* 1983). In particular both this and previous studies have consistently found that the initial target for NO_2 toxicity is the epithelium in the terminal bronchiole and proximal alveoli.

In addition areas of focal haemorrhage and damage to alveolar capillaries, similar to effects recently reported in the guinea-pig (Azouley-Dupois *et al.* 1983), were seen in the hamster but not in the rat lung. Pulmonary and vascular changes have also been induced by NO_2 in the beagle dog, but only after bypassing the upper respiratory tract (Guidotti 1980), whereas much greater concentrations (50 parts/10^6) in intact dogs failed to produce significant pulmonary effects (Carson *et al.* 1962). The geometry of the respiratory tract of the hamster and rat differ significantly from one another (Schlesinger & McFadden 1981) and it may be that a lower concentration of NO_2 reaches the lower respiratory tract in the rat than in the hamster (Brain 1970). This, however, does not appear to be the only factor that renders the hamster more susceptible to the acute effects of NO_2.

A further indication of NO_2-induced pulmonary injury in the hamster, but not in the rat, was the infiltration of the alveolar lumen by both polymorphonuclear leucocytes and lymphocytes as well as macrophages. As a consequence activation of alveolar macrophages was evident as the macrophages had increased in size, spread more extensively on glass cover slips and exhibited an increased ability to phagocytose SRBC. These findings may relate to the enhancement of immune function reported by Holt *et al.* (1979), after short-term, but not chronic exposure to NO_2. On the other hand, little difference in lavaged cell populations was observed in NO_2-exposed rats compared to control animals, and exposure was associated with a significantly decreased ability of rat alveolar macrophages to form rosettes with sensitized SRBC. The effects in the rat thus appear similar to those reported in the rabbit (Acton & Myrvik 1972) where NO_2 has been shown to have an inhibitory effect on macrophage phagocytosis and consequent microbiocidal activity (Amoruso *et al.* 1981). It may also be that any toxic effects of NO_2 on hamster alveolar macrophages are masked by the early inflammatory response.

Whilst structural lung changes are indicators of a direct toxic effect, other lung changes are thought to be protective mechanisms against the deleterious consequences of lipid peroxidation or other oxidative membrane damage caused by NO_2 exposure (Sagai *et al.* 1982; Blank *et al.* 1978; Blank *et al.* 1982; Wright & Mavis 1981). These include increased biosynthesis of whole lung surfactant phospholipids and direct stimulation of enzyme activity in lung cell populations. In the present study increases in total lung surfactant levels were evident in both species after NO_2 exposure, but only in the hamster was this paralleled by an appreciable increase of extracellular surfactant. Increased levels of total lung surfactant in the rat did not lead to increased availability of functional surfactant in the airway. It may be that a specific protection enzyme such as glutathione peroxidase, glutathione reductase or glucose-6-phosphate dehydrogenase present in higher base levels in the rat (Sagai *et al.* 1982) compared to the hamster (De Nicola *et al.* 1982) provide

sufficient resistance to the acute toxic effects of NO_2. The cells thought to be responsible for the production of these enzymes in the lung are the type II alveolar cells and the Clara cell (Witchi & Cote 1977), and a comparison of both cell types in the two species has shown major morphological differences in the Clara cells, even though the type II cells are very similar. The Clara cell in the hamster has much less rough endoplasmic reticulum than the corresponding cell in the rat (Plopper *et al.* 1980) and its ability to synthesize rapidly new enzymes may thus be more limited. The observations in this study that many Clara cells were seen in mitosis in the hamster, but not in the rat lung may relate to the detoxifying role of the Clara cell. Alternatively the mitotic activity of the cells could be associated with the secretory nature of the Clara cell (Yoneda & Birk 1981). Whilst synthesis of surfactant phospholipids has been accepted as occurring primarily in the type II alveolar cell some authors have postulated a role for the Clara cell in surfactant production (Yoneda 1978; Widdicombe & Pack 1982).

In the present study of the increased levels of extracellular surfactant found in the hamster lung after NO_2 exposure did not appear to be the results of increased number or activity of type II cells. Thickening of the alveolar epithelium was not observed, nor was there any observable alteration in size or numbers of lamellar bodies within the type II cells, indicative of secretion of preformed surfactant from storage granules. It is conceivable therefore, that the Clara cell may contribute to or have an effect on airway surfactant levels at least in this species.

In the rat lung, on the other hand, where no appreciable increase of extracellular surfactant was found, electron microscope examination of the tissue sections indicated substantial intracellular accumulation of surfactant and an increased number of type II cells in mitosis. These differences in response may be related to the differences in short- and long-term effects of NO_2 in these species.

This study has served to demonstrate that the hamster and the rat react differently to the same dose of nitrogen dioxide. It is evident that either the two species are not receiving identical doses in equivalent regions of the lung and/or that the biochemical protective mechanisms differ between the two. The results are particularly relevant when applied to inhalation toxicology where single species studies are often intended to determine safe levels for man despite good evidence to suggest that both lung geometry and biochemistry will significantly affect results.

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